Genetics of Pink Bollworm Resistance to *Bacillus thuringiensis* Toxin Cry1Ac

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ABSTRACT Laboratory selection increased resistance of pink bollworm (*Pectinophora gossypiella*) to the *Bacillus thuringiensis* toxin Cry1Ac. Three selections with Cry1Ac in artificial diet increased resistance from a low level to >100-fold relative to a susceptible strain. We used artificial diet bioassays to test F_1 hybrid progeny from reciprocal crosses between resistant and susceptible strains. The similarity between F_1 progeny from the two reciprocal crosses indicates autosomal inheritance of resistance. The dominance of resistance to Cry1Ac depended on the concentration. Resistance was codominant at a low concentration of Cry1Ac, partially recessive at an intermediate concentration, and completely recessive at a high concentration. Comparison of the artificial diet results with previously reported results from greenhouse bioassays shows that the high concentration of Cry1Ac in bolls of transgenic cotton is essential for achieving functionally recessive inheritance of resistance.

KEY WORDS *Pectinophora gossypiella*, *Bacillus thuringiensis*, Cry1Ac, transgenic cotton, inheritance, resistance management

GENETICALLY ENGINEERED CROP plants that express toxin genes from *Bacillus thuringiensis* (Bt) are becoming increasingly important for insect pest management (Schnepf et al. 1998). Transgenic cotton producing Bt toxin Cry1Ac (Bt cotton), which kills larvae of some of the key lepidopteran pests of cotton, was grown on >1 million hectares in the United States in 1998 (James 1998). The greatest threat to the continued success of Bt cotton and other Bt crops is evolution of resistance by pests (Tabashnik 1994a, Gould 1998, Frutos et al. 1999). Strains of at least 10 insect species have evolved resistance in the laboratory to Bt toxins; diamondback moth populations in many regions have evolved resistance in the field to sprays of formulated Bt (Tabashnik 1994a, Frutos et al. 1999).

Understanding the genetic basis of resistance is essential for designing strategies to extend the efficacy of Bt toxins in transgenic crops. Current management efforts focus on the refuge strategy, which is mandated by the United States Environmental Protection Agency to delay evolution of resistance to Cry1Ac by lepidopteran pests of cotton. The refuge strategy is based primarily on computer simulations of one-locus genetic models and limited empirical data from small scale tests (Georghiou and Taylor 1977; Tabashnik and Croft 1982; Tabashnik 1994b; Liu and Tabashnik 1997a; Roush 1997; Gould 1998, 2000; Gould and Tabashnik 1998; Shelton et al. 2000). For cotton growers, refuges consist of cotton that does not produce Cry1Ac (non-Bt cotton) grown in or near Bt cotton fields. In principle, susceptible moths emerging from refuges will mate with resistant moths from Bt cotton fields. Under ideal circumstances, their hybrid F_1 progeny will be killed by Bt cotton and resistance evolution will be delayed substantially. Thus, the refuge strategy is expected to work best if resistance to Bt cotton is inherited as a recessive trait. In strict genetic terms, recessive inheritance means that the phenotype of susceptible homozygotes is indistinguishable from that of heterozygotes individuals. In practice, the critical question is whether resistance is functionally recessive, which means that the concentration of toxin in Bt cotton is sufficiently high that the mortality of heterozygotes is equal to or nearly equal to the mortality of susceptible homozygotes.

Most analyses of the dominance of resistance to Bt toxins have been based on bioassays in which resistant larvae, susceptible larvae, and their hybrid F_1 progeny are tested against a series of concentrations of a single toxin or of a formulation of several toxins (Tabashnik 1994a, Frutos et al. 1999). Results from such tests show that resistance to Bt toxins is partially to completely recessive in most but not all cases (Tabashnik et al. 1998, Frutos et al. 1999). If the extent of recessiveness varies as a function of the concentration of toxin (e.g., Liu and Tabashnik 1997b), one must perform bioassays on the Bt crops themselves to address the critical issue of functional recessiveness. However, we know of only three cases in which inheritance of resistance to Bt crops has been reported. Resistance of diamondback moth, *Plutella xylostella* (L.), to Bt broccoli

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(Metz et al. 1995) and tobacco budworm, *Heliothis virescens* (F.), to Bt cotton is recessive (Gould et al. 1997). For pink bollworm, *Pectinophora gossypiella* (Saunders), a major pest of cotton in the southwestern United States (Ingram 1994, Henneberry and Naranjo 1998), greenhouse studies with neonates infesting bolls on live plants showed that resistance to Bt cotton is recessive (Liu et al. 1999).

Here we report analysis of the genetic basis of resistance to Bt toxin Cry1Ac based on artificial diet bioassays with a laboratory-selected strain of pink bollworm. This approach enabled us to evaluate dominance across a series of concentrations of Cry1Ac and to compare results from artificial diet with previous results from Bt and non-Bt cotton plants (Liu et al. 1999). Further, we report here the origins of the resistant strain (APHIS-98R) and its response to selection.

Materials and Methods

Insect Strains and Rearing. We started with two strains of pink bollworm: APHIS-S and APHIS-BTX. APHIS-S is a susceptible strain that had been reared in the laboratory for >20 yr. APHIS-BTX was derived from APHIS-S and infused with individuals from a strain that had been derived more recently from the field (Bartlett 1995). Before the current study, APHIS-BTX had been exposed repeatedly to artificial diet containing leaf powder from Bt cotton and had evolved a low level of resistance to Cry1Ac (Bartlett 1995). In 1998, we derived a highly resistant strain, APHIS-98R, from the APHIS-BTX strain by selecting a subset of APHIS-BTX with Cry1Ac as described below (see *Selection* section). Bartlett (1995) referred to APHIS-S as APHIS and APHIS-BTX as BTX. We use the acronym APHIS as a prefix in the name of all three strains to emphasize their common origin; the characters following APHIS identify the specific strain.

We reared larvae on artificial wheat germ diet (USDA, Phoenix, AZ), using a modification of the method of Bartlett and Wolf (1985). Pieces of paper towel laden with eggs were put on aluminum foil (to prevent wetting and mold) on cubes of diet in paper cups (400 ml). The cups were sealed with a screened lid lined with four layers of tissue to provide ventilation and prevent escape of larvae. The cups were put in plastic containers lined with hexcel (honeycomb-shaped cardboard) at the bottom to provide pupation sites for mature larvae that bored out of cups. The plastic containers were sealed with screened lids and kept in environmental chambers. Pupae were collected and held in paper cups with screened lids for adult emergence. Adults were provided with 10% honey water in an inverted vial (2 ml) inserted through a hole in the lid of each cup. As oviposition substrate, a piece of paper towel was held on top of the screened lid of each cup with a metal washer. Eggs were collected every two to 3 d. Rearing and bioassays were done at 27°C and a photoperiod of 14:10 (L:D) h.

Incorporation of *B. thuringiensis* Toxin in Diet. We used liquid formulation MVP II Bioinsecticide (Dow Agrosciences, San Diego, CA), which contains 20% Cry1Ac protoxin expressed in and encapsulated by transgenic *Pseudomonas fluorescens.* For brevity, we refer to MVP II Bioinsecticide as Cry1Ac.

Cry1Ac was mixed into artificial diet using a food processor. The appropriate concentrations of Cry1Ac diluted with distilled water were added to artificial diet at 1 ml/100 g diet to achieve the desired concentrations in the diet. The diet was then blended for about 10 s. After each of three such blendings, diet was scraped from the side of the food processor to ensure thorough mixing. Trials of this process with dye indicated that thorough mixing was achieved.

Selection. The APHIS-98R strain was selected for resistance by rearing larvae on diet containing 10, 20, and 20 μg Cry1Ac per gram of diet, respectively, in three consecutive generations. Pupae from each selection were collected and adult survivors were reared to produce progeny to initiate the next generation. All tests of APHIS-98R were conducted after three generations of selection were completed.

Bioassay. To measure the effect of Cry1Ac on survival, we tested groups of five neonates on 10–12 g of diet per cup in plastic cups (37.5 ml) (Bio-Serv, Frenchtown, NJ). After neonates were added, we sealed each cup by gluing on a paper lid to prevent larvae from escaping. For each strain and each concentration of Cry1Ac tested, eight cups with five neonates per cup were held in an environmental chamber for 21 d. After 1 wk, cups were put in a sealed plastic box with a small screened window in the lid for ventilation and a cup of water to maintain moisture. The boxes enabled us to collect mature larvae that bored out of cups. At 21 d, the number of survivors and their stage of development were recorded. Pupae and live fourth instars were counted as survivors.

Response to Selection. To measure the response of APHIS-98R to selection, we tested APHIS-S, APHIS-BTX, and APHIS-98R at generation 4 with the survival bioassay on diet containing 0 (control), 0.1, 1.0, and 10 μg Cry1Ac per gram of diet. At each concentration, 40 neonates from each strain were tested. A total of 160 neonates was tested for each strain.

Maternal Effects, Sex Linkage, and Dominance. To evaluate maternal effects, sex linkage, and dominance we used the survival bioassay to test F_1 offspring from each of the reciprocal crosses between APHIS-98R and APHIS-S. We determined the sex of pupae visually. For one cross, we pooled 50 APHIS-S female pupae and 50 APHIS-98R male pupae. For the other cross, we pooled 50 APHIS-98R female pupae and 50 APHIS-S male pupae. We tested neonates of F_1, APHIS-S, and APHIS-98R on diet containing 0 (control), 1.0, and 10 μg Cry1Ac per gram of diet. A total of 40 neonates was tested in eight bioassay cups at each Cry1Ac concentration for each strain and each of the two sets of reciprocal F_1 offspring.

Data Analysis. Survival of treated larvae was calculated as unadjusted survival of treated larvae (%) divided by survival of untreated (control) larvae (%).

Table 1. Responses of pink bollworm larvae to Cry1Ac in artificial diet bioassays

Concn (μg/g)	Strain	Survival ± SE (%)
0.1	APHIS-S	50.0 ± 6.9b
	APHIS-BTX	59.4 ± 11.5b
	APHIS-98R	95.1 ± 3.2a
1.0	APHIS-S	2.9 ± 2.9b
	APHIS-BTX	15.6 ± 9.4b
	APHIS-98R	72.7 ± 6.5a
10.0	APHIS-S	0b
	APHIS-BTX	0b
	APHIS-98R	73.1 ± 10.4a

Survival was divided by the survival in controls without Cry1Ac. Data were transformed by arcsine$\sqrt{x}$ before analysis. For each concentration, values followed by the same letter were not significantly different, Ryan–Einot–Gabriel–Welsch multiple range test, $P > 0.05$ (SAS GLM procedure, SAS Institute 1985).

We compared survival among strains at each concentration of Cry1Ac using the Ryan–Einot–Gabriel–Welsch multiple range test (SAS Institute 1985). We estimated dominance of resistance (h) with the single-concentration method (Liu and Tabashnik 1997b). Values of h range from 0 (completely recessive) to 1 (completely dominant) and a value of 0.5 indicates codominant resistance.

Results

Response to Selection. Selection with Cry1Ac increased resistance of APHIS-98R to Cry1Ac. After three rounds of selection, APHIS-98R had significantly higher survival than either APHIS-S or APHIS-BTX at all three concentrations of Cry1Ac tested (Table 1). APHIS-S larvae had 50.0% mortality at a concentration of 0.1 μg Cry1Ac per gram of diet. In the same set of tests, 10 μg Cry1Ac per gram of diet killed only 26.9% of APHIS-98R (Table 1). Thus, the results in Table 1 imply that the LC_{50} for APHIS-98R was >100-fold greater than the LC_{50} for APHIS-S. At each concentration tested, survival did not differ significantly between APHIS-S and APHIS-BTX (Table 1).

Maternal Effects, Sex Linkage, and Dominance. Resistance to Cry1Ac was autosomally inherited. Bioassays of F_1 progeny from crosses between APHIS-98R and APHIS-S showed no significant differences in survival between the two reciprocal crosses (Table 2). Thus, maternal effects and sex linkage were not evident.

Dominance of resistance to Cry1Ac in APHIS-98R varied with the concentration of Cry1Ac (Table 2). At the lowest concentration tested (0.1 μg Cry1Ac per gram of diet), mortality of APHIS-S larvae was 57.6% and resistance was codominant ($h = 0.53$). At an intermediate concentration (1.0 μg Cry1Ac per gram of diet), mortality of APHIS-S was 100% and resistance was partially recessive ($h = 0.23$–0.26). At the highest concentration tested (10 μg Cry1Ac per gram of diet), mortality of APHIS-S was 100% and resistance was completely recessive ($h = 0$).

Results from the inheritance tests confirm that APHIS-98R had >100-fold resistance relative to APHIS-S. In this set of tests, 0.1 μg Cry1Ac per gram killed 57.6% of APHIS-S larvae, but a concentration 100 times greater (10 μg Cry1Ac per gram of diet) killed only 40.6% of APHIS-98R larvae (Table 2).

Table 2. Dominance *(h)* of resistance to Cry1Ac in pink bollworm larvae in artificial diet bioassays

Concn (μg/g)	Strain	Survival ± SE (%)[a]	Fitness	h
0.1	APHIS-98R	90.6 ± 4.6a	1	
	APHIS-S	42.4 ± 8.9b	0.47	
	F_1a	68.4 ± 8.6ab	0.75	0.53
	F_1b	67.7 ± 8.2ab	0.75	0.53
1.0	APHIS-98R	78.1 ± 7.4a	1	
	APHIS-S	0c	0	
	F_1a	17.7 ± 5.9b	0.23	0.23
	F_1b	20.6 ± 5.3b	0.26	0.26
10.0	APHIS-98R	59.4 ± 8.1a	1	
	APHIS-S	0b	0	
	F_1a	0b	0	0
	F_1b	0b	0	0

For each strain, 40 neonates were tested in eight cups at each concentration of Cry1Ac. F_1a was offspring of cross between APHIS-98R females and APHIS-S males. F_1b was offspring of cross between APHIS-98R males and APHIS-S females. Data were transformed by arcsine $\sqrt{x}$ before being analyzed. For each concentration, values followed by the same letter were not significantly different, Ryan–Einot–Gabriel–Welsch multiple range test, $P > 0.05$ (SAS GLM procedure, SAS Institute 1985).

[a] Only live 4th instars and pupae at 21 d after the start of the bioassay were counted as survivors.

Discussion

Pink bollworm strain APHIS-98R responded quickly to selection with Cry1Ac. After only three rounds of selection, APHIS-98R attained >100-fold resistance relative to a susceptible strain (Table 1). This response indicates that resistance alleles were not rare in the parent strain APHIS-BTX, which had been selected repeatedly with Cry1Ac in the laboratory (Bartlett 1995). Yet, resistance was not readily detected in bioassays of APHIS-BTX, as reflected in the lack of significant differences in survival between APHIS-BTX and APHIS-S (Table 1). A similar response to selection with Cry1Ac occurred in the Arizona pooled resistant strain of pink bollworm (AZP-R), which was derived independently from individuals collected in 1997 from nine Arizona cotton fields (Patin et al. 1999, Tabashnik et al. 2000).

In the southwestern United States, where pink bollworm is a major pest of cotton, it can complete four to five generations a year (Henneberry and Naranjo 1998). The response to selection in the laboratory suggests that pink bollworm has the potential to increase its resistance to Cry1Ac toxin from a barely detectable level to over 100-fold in a single growing season. So far, however, Bt cotton has remained extremely effective against pink bollworm (Simmons et al. 1998, Patin et al. 1999, Tabashnik et al. 2000). Differences between the laboratory and field, including the presence of non-Bt cotton refuges in the field, may slow evolution of resistance in the field.

Although the dominance of resistance to Cry1Ac in APHIS-98R varied with the concentration of Cry1Ac in bioassays with artificial diet, previously reported results showed that on Bt cotton plants in the greenhouse, APHIS-98R resistance was recessive (Liu et al. 1999). Adjusted survival of APHIS-98R was 37% on Bt cotton bolls (Liu et al. 1999) compared with 59–73% at 10 μg Cry1Ac per gram of diet in the artificial diet bioassays (Tables 1 and 2). These results suggest that the toxicity of Cry1Ac in the bolls of Bt cotton tested in the greenhouse (Liu et al. 1999) was greater than that of 10 μg Cry1Ac per gram of diet in the artificial diet bioassays. The greenhouse tests with Bt cotton (Liu et al. 1999) provide direct evidence that APHIS-98R resistance to Bt cotton is functionally recessive. The comparison with artificial diet bioassays suggests that at a concentration of Cry1Ac that is comparable to or slightly less than that of Bt cotton bolls, resistance was also recessive. Thus, the greenhouse and laboratory bioassays imply that resistance to Bt cotton is recessive, which is favorable for the refuge strategy. The dependence of dominance of resistance on toxin concentration in artificial diet bioassays indicates that a high concentration of Cry1Ac in Bt cotton is essential to kill hybrid F_1 offspring.

Comparisons with the artificial diet results reported here clarify a previous report (Liu et al. 1999) of anomalously high adjusted survival of APHIS-S (6%) and F_1 hybrid progeny (2%) on bolls of Bt cotton in the greenhouse. In contrast to the greenhouse results, adjusted survival at 10 μg Cry1Ac per gram of diet in the artificial diet bioassays was 0% for APHIS-S and F_1 (Tables 1 and 2). As noted above, mortality of APHIS-98R was greater in greenhouse tests with Bt cotton than it was in laboratory tests with 10 μg Cry1Ac per gram of diet. We suspect that in the greenhouse, some APHIS-98R larvae moved from their assigned plants to Bt cotton plants that were supposed to be infested only with larvae from APHIS-S or F_1, thereby inflating survival recorded for APHIS-S and F_1. In the laboratory, larvae could not move between sealed bioassay cups and only APHIS-98R larvae survived at 10 μg Cry1Ac per gram of diet. Further, we recovered one of the putative APHIS-S individuals that survived on a boll of Bt cotton and mated it with a resistant individual. The progeny were resistant, indicating that both parents were homozygous for resistance. Finally, field counts of pink bollworm larvae in 218,150 bolls of Bt cotton and adjacent non-Bt cotton imply that survival of susceptible larvae on Bt cotton from 1995 to 1998 was $<0.7\%$ (Tabashnik et al. 2000).

In summary, the results with artificial diet reported here confirm the previously reported conclusion that resistance of the APHIS-98R strain of pink bollworm to Cry1Ac in Bt cotton is inherited as a functionally recessive trait. The results reported here also indicate that the resistance of APHIS-98R to Cry1Ac is >100-fold relative to the susceptible strain APHIS-S. These results on inheritance and level of resistance fit the predominant pattern of lepidopteran resistance to Cry1A toxins, termed "mode 1" resistance to Bt (Tabashnik et al. 1998). The results with APHIS-98R reported here and previously (Liu et al. 1999) also imply that one key assumption of the refuge strategy, recessive inheritance, is valid for pink bollworm resistance to Bt cotton.

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